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Microtiter plate-sized standalone chip holder for microenvironmental physiological control in gas-impermeable microfluidic devices†

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We present a microtiter plate-sized standalone chip holder for precise control of physiological conditions inside closed microfluidic cell culture systems, made from gas-impermeable materials. Specifically, we demonstrate the suitability of the holder to support cell growth in a glass chip, to allow time-lapse imaging of live cells and the creation of a hypoxic environment, all relevant for applications in regenerative medicine research.

Microfluidic systems are successfully applied to create tailored chemical and physical microenvironments with high fidelity at the micrometre scale, enabling in-depth analysis of cell behaviour, in a milieu that mimics their natural biological environment.¹ As such, microfluidic systems have become highly relevant tools for fundamental (cell) biological studies, diagnostics, and pharmacological screens. Furthermore, as we recently discussed, microfluidic systems may become widely used in regenerative medicine (RM) research.² Microfluidics based *in vitro* systems for RM research should allow for tailoring of the biological environment by controlling gas-exchange, temperature, pH, nutrient supply, and metabolite removal. Another important aspect to be taken into account when developing such a system is the possibility to perform continuous and real-time assays, for example, based on live or time-lapse imaging, increasing therewith the throughput of screening. Finally, such systems are preferably compatible with standard laboratory equipment, such as microscopes and plate readers.

The majority of currently applied microfluidic systems intended for biological studies are made from polydimethylsiloxane (PDMS). PDMS has the advantage of being gas-permeable, and therefore cells grown in PDMS based systems can be cultured using conventional laboratory incubators.^{3–5} Furthermore, PDMS is relatively inexpensive and its fabrication is straightforward.⁶ The use of PDMS is, however, also

associated with important drawbacks, such as limited fluidic control due to the porous nature of the material and unpredictable and unstable surface chemistry.⁷ Furthermore, severe concerns are shared by many biologists regarding the incomplete biological characterization of the effects of this material.⁷ Other materials that are better characterized from this perspective, such as glass and polystyrene (PS), owing to their widespread use in conventional *in vitro* systems, exist.⁷ However, they are not or poorly gas-permeable, which limits their applicability to closed cell-culture systems, where CO₂ and O₂ must be tightly regulated. Closed *in vitro* systems are of particular importance in the RM research, where, among others, interactions of cells with various natural and synthetic biomaterials are studied, the majority of which are gas-impermeable.^{2,8} Such closed systems require alternative ways to control physical parameters inside the cell culture device, for example, in the form of a standalone incubator.

Current commercially available standalone culture systems, such as microscope stage incubators (*e.g.* Okolab, Pecon), are bulky, limiting control of the microenvironment. In addition, they require modifications of the microscope, and they are dedicated, not portable, and do not readily accommodate microfluidic connections. Systems that facilitate microfluidic connections do not allow for control of temperature and gas conditions in closed systems (*e.g.* Micronit, microLIQUID) or are mainly meant for PDMS cell culture devices.^{9–11}

To overcome these issues, we have developed a platform in which entirely closed microfluidic systems made from gas-impermeable materials can be used for cell culture under varying physiological conditions. The complete system consists of a glass microfluidic chip inserted into a microtiter plate-sized chip holder, which also functions as a standalone incubator, allowing gas-tension and temperature control. The system is suitable for *in situ* time-lapse or live imaging of

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cells and is compatible with standard laboratory (imaging) equipment. Control on the gas composition inside the glass cell culture chip is achieved by using gas-permeable tubing inside the closed chip holder. As such, the system supports cell growth, but it can also be used to mimic deviating physiological conditions, such as oxygen deprivation, also known as hypoxia.

Chip holder

The chip holder consists of an aluminium chamber, into which the chip can be inserted, allowing for fluidic interfacing using a manifold connected to an array of capillaries. The whole platform was designed, fabricated and assembled in-house. Aluminium was chosen for its high thermal conductivity and machinability. The whole aluminium chamber is used as a heat sink and is kept at a stable temperature using two square 20 mm Peltier elements (MULTICOMP, Farnell, The Netherlands) placed on either side of the chamber, and each contains an attached outer heat sink (Fig. 1A). The chamber and heat sinks are thermally insulated using polyoxymethylene (POM) spacers. Since the whole device is precision milled, it is fully symmetrical and involves a relatively small temperature difference ($37 - RT = 12$ K), with negligible temperature gradient over a distance of 1 mm, as measured using conventional probes ($< \pm 0.05$ °C). To ensure stable temperature and fast response, the Peltier elements are powered by a computer programmable controller, capable of delivering pulse-width modulated and polarizable output (TCM, Electro Dynamics Ltd., Southampton, United Kingdom). The feedback temperature sensor (NTC) is placed on the side of the chip (Fig. 1B–C) so as not to block the light, and an offset of 0.8 K was determined using an external temperature probe near the micro-chamber. This resulted in a stable temperature at a desired value (37 °C) with a tested SD of ± 0.003 °C over a period of 20 h. This feature is particularly important for the stability of the cell culture and imperative for the stability and predictability of the fluidics.

Gas exchange is ensured through the microfluidic connections with the chip inside the closed chip holder, using perfluoroalkoxy (PFA) capillaries (150 μ m ID and 360 μ m OD PFA HP Plus, DuPont, USA), each having an internal volume of 6 μ L (Fig. 1B–C). By controlling the flow rate through the tubing, the residence time of the medium inside the tubing can be set to ensure that gas equilibrium is reached before the medium enters the chip (for detailed calculation to determine minimum residence time, please see ESI†). Fused silica capillaries (200 μ m ID and 360 μ m OD, PolyMicro, France) are used for outlet connections and collection of (waste) medium, since the medium is passed only once. Even though fluoropolymers are not known for their inherent gas-permeability, they provide a good compromise between chemical resistance, thermal stability and gas exchange. The gas mixture is provided by 5% CO₂ supplemented compressed air (Linde Group, The Netherlands) and reduced oxygen mixture is obtained by partial mixing with N₂. To provide sufficient humidity, to avoid possible evaporation through the PFA tubing

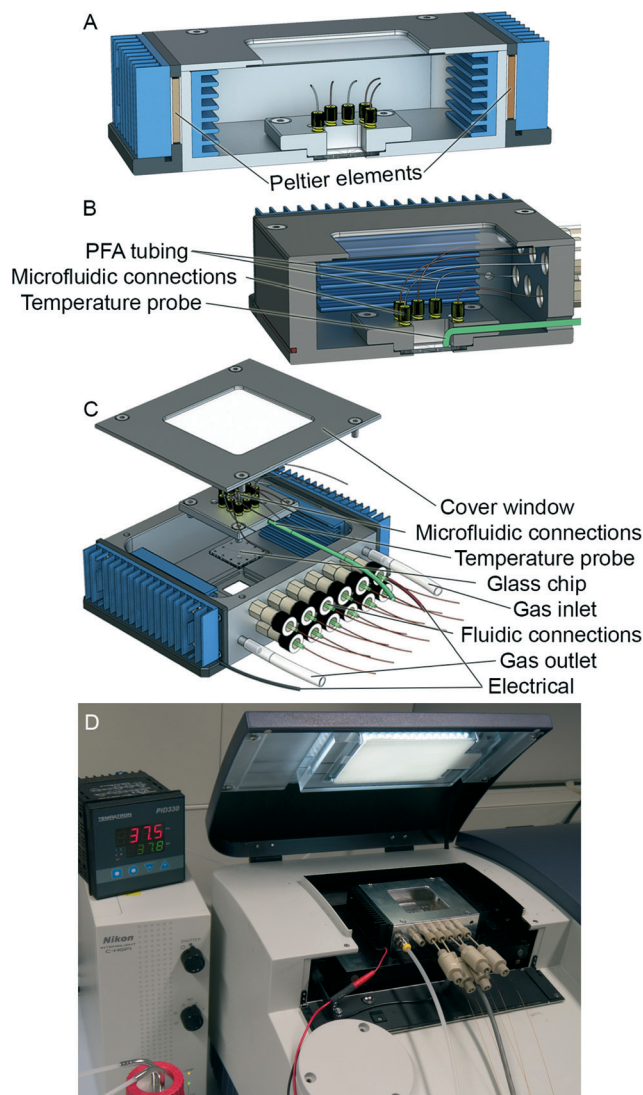


Fig. 1 (A) Schematic depiction of a longitudinal cut-through of the chip-holder, showing the position of the two Peltier elements and heat sinks. (B) Depiction of a transverse cut-through, showing the position of the temperature probe, the chip, and its connections to the gas-permeable PFA tubing. (C) Exploded view, showing the different separable parts. The lid includes a glass window for top illumination for contrast microscopy. (D) Picture of the chip holder inside a microtiter plate microscope with electrical, gas and fluidic connections. The bottle in the bottom left corner is the bubbler to keep the humidity high to prevent evaporation through the gas-permeable tubing.

(Fig. 1D), the gas mixture is flown through a water bubbler before entering the chip holder.

The whole platform has the footprint of a microtiter plate (127.5 mm \times 85.5 mm) and a height of 32 mm. These dimensions make it compatible with a variety of read-out equipment meant for microtiter plates. For live imaging, the chip holder is placed in a microtiter plate microscope (BD Pathway 435, USA) capable of fully automated fluorescence and bright-field microscopy (Fig. 1D) or in a fully automated DIC/confocal microscope (NIKON Ti-Eclipse with A1 confocal, Japan).

Glass chips

Two different chip designs were tested to demonstrate the effectiveness of the platform: (i) a device with two $650\ \mu\text{m} \times 4\ \text{mm}$ rectangular chambers connected to two supply channels (Fig. 2A), *via* an array of $1\ \mu\text{m} \times 3\ \mu\text{m} \times 10\ \mu\text{m}$ channels; and (ii) a device with a $650\ \mu\text{m}$ square chamber surrounded by four supply channels (Fig. 2C), also connected to the chamber by a similar array of microchannels (Fig. 2D). Thereby, diffusion is dominant over convection between the channel and the chamber due to the high relative resistance of the connecting channels. This configuration allows for shear stress-free culture of cells, which is essential for delicate human cells.

The microdevices were fabricated in glass, as described in detail in the ESI†. In short, the microfluidic features of the chip were fabricated in $0.21\ \text{mm}$ glass, using an elaborate two-step wet etching process to successfully create structures with two different heights. Thereafter, the glass substrates were thermally bonded with $1.1\ \text{mm}$ glass substrates with powder blasted via-holes. Finally, devices were diced in $2\ \text{cm}$ square dies (Fig. 2B).

Cell culture

To demonstrate the possibility to perform live or time-lapse imaging, murine myoblastic C2C12 cells were cultured over 2 days and imaged using an automated DIC microscope (NIKON Ti Eclipse). C2C12 cells were cultured in Dulbecco's-Minimum Essential Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), $100\ \text{mg}\ \text{mL}^{-1}$ penicillin and $100\ \text{mg}\ \text{mL}^{-1}$ streptomycin (Pen/Strep, Invitrogen). After expansion culture, they were dissociated using trypsin (Invitrogen), resuspended to a concentration of approximately $5\ \text{million cells mL}^{-1}$, and injected into the chip using a syringe through the cell loading channels into the

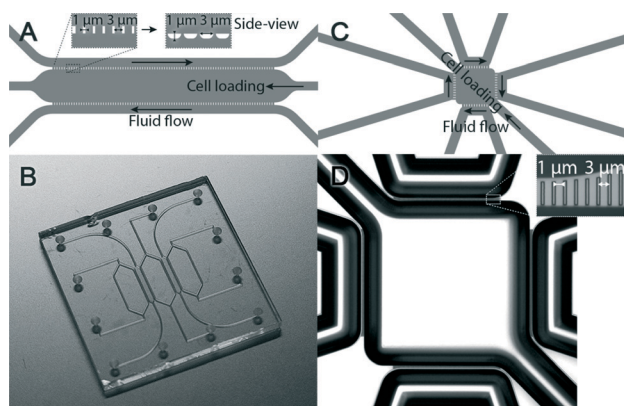


Fig. 2 (A) Schematic diagram of the rectangular chamber, showing the flow directions in the supply channels and cell loading channels, with an enlargement and side-view of the small channel array shown as inset. (B) Photograph of the $2\ \text{cm}$ square die with two rectangular chambers. (C) Schematic diagram of the square chamber. (D) Bright-field microscopy image of a glass chip, depicting the chamber with the surrounding supply channels, with an enlargement of the smaller channels that connect the supply channel with the chamber shown as inset.

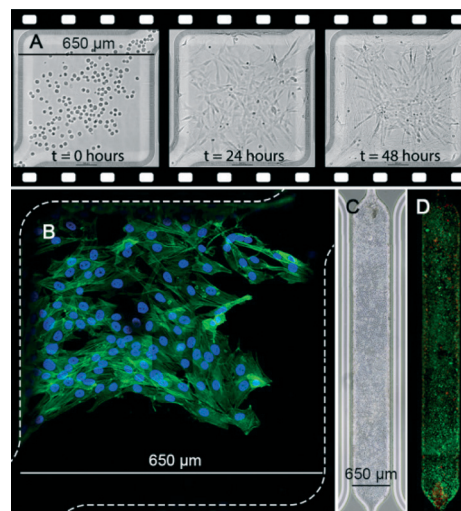


Fig. 3 (A) Bright-field microscopy images taken from 2 day time-lapse series of C2C12 cells at different time points, with $t = 0$ taken just after cell loading. (B) Confocal fluorescence microscopy image of MG63 cells in the square chamber after 2 days of proliferation, with stained nucleus (DNA, DAPI, blue) and cytoskeleton (F-actin, Phalloidin, green). (C) Bright-field microscopy stitched image of the rectangular chamber with Cho cells after 5 days of culture. (D) Epi-fluorescence image of the same Cho cells with live/dead staining, live cells being green and dead cells being red.

chamber. Cells were left to proliferate for 2 days, while flowing cell culture medium in the supply channels (single pass), at a flow rate of $8\ \mu\text{L h}^{-1}$ using a precision syringe pump (Nexus Performance, Chemyx, USA). Fig. 3A presents snapshots taken from a 2 day time-lapse series on the growth of C2C12 cells in the square chamber at different time points: at time point 0, just after loading of the cells, and at time points 22 and 48 h, where the attachment and spreading of cells can be observed, as well as the formation of a cell monolayer.

To demonstrate culture of more delicate human cells, osteoblastic MG-63 cells were cultured for 2 days, subsequently fluorescently stained with Phalloidin (cytoskeleton, F-actin) and DAPI (nuclei), and imaged by a fluorescence confocal microscope (NIKON Ti Eclipse with A1). MG-63 cells were cultured in αMEM supplemented with 10% FBS and Pen/Strep. After culture, cells were rinsed with phosphate buffered saline (PBS, Invitrogen) and fixed with 4% para-formaldehyde (PFA, Sigma-Aldrich) for 10 min, before being stained *in situ* by flushing the staining solutions through the chamber *via* the cell loading channels. As shown in Fig. 3B, MG63 cells were successfully stained inside the chip, which demonstrates the suitability of the platform for *in situ* cell staining and fluorescence imaging.

Viability assay

Cell viability was assessed using a standard live/dead assay on a Chinese hamster ovary (Cho) cell line, cultured in DMEM with 10% FBS and Pen/Strep. All channels and the chamber were first flushed with PBS, and subsequently a mixture of

2 μM calcein AM and 4 μM ethidium homodimer-I (EthD-I, Invitrogen), to stain live and dead cells, respectively, was introduced into the chamber *via* the cell loading channels and left for 20 min, before washing again with PBS. Samples were imaged using an epi-fluorescence microscope (TS100, NIKON, Japan), and overview images were created by stitching multiple images together. After 5 days of culture, in the rectangular culture chamber, the large majority of cells were found viable, with a few dead cells homogenously spread through the chamber (Fig. 3C–D), similar to conventional cell culture, proving that cells could be kept viable inside the platform for multiple days. Due to the seeding method, the entrance and exit of the chamber show slightly more red fluorescence due to cell debris flowing back from the dead-volume of the loading channels.

Hypoxia assay

Oxygen is a critical nutrient for cell survival, which is likely the first one to become limited due to its high uptake and low solubility. Therefore, it is frequently used as a model nutrient.¹² When cells are subjected to low oxygen conditions (hypoxia), numerous genes are up- or down-regulated like angiogenesis related genes to promote cell survival.¹³ To study this, a Cho cell line (Cho HRE-GFP cells) transfected with a green fluorescence protein (GFP) hypoxia reporter was employed to demonstrate the ability of our microfluidic platform to recapitulate normal and hypoxia conditions. The cells fluoresce in case of limited availability of oxygen. More specifically, hypoxia-inducible factor-1 (HIF-1) translocates into the nucleus and binds to the hypoxia responsive element (HRE) promoter, thereby activating GFP transcription (for details on transfection and clonal procedure, please see the earlier work by Liu and co-workers).¹³

Cho cells were cultured in the chip, under normal (20%) and reduced (4%) oxygen conditions inside the chip holder, using regular culture medium supplied at a flow rate of 1 $\mu\text{L h}^{-1}$

through the supply channels. Under these conditions, the residence time of 6 h was sufficient for the medium to reach equilibrium through the PFA tubing. The cells were introduced in the chamber by the cell loading channels and left to attach under normoxic conditions for 8 h. Cell culture either continued at 20% O_2 , or the culture condition was switched to hypoxic, whereby the gas mixture flowing in the chip holder contained approximately 4% O_2 . After only 22 h of culture under hypoxia, cells showed increased expression of green fluorescence protein (Fig. 4D–F), demonstrating that by varying the oxygen level inside the chip holder, cells in the culture chamber of the chip are affected. In contrast, after 48 h of culture in normoxia, limited or no green fluorescence was observed (Fig. 4A–C), showing that under normal conditions, the cells inside the gas-impermeable glass chip have sufficient amount of oxygen not to turn to a hypoxic state. These results showed that the platform presented here can be utilized in a standalone fashion with chips made of gas-impermeable materials and that gas composition inside the chip can be varied for mimicking specific *in vivo* conditions, like hypoxia.

Conclusions

Here we have presented a standalone chip holder, particularly meant for microfluidic platforms made from gas-impermeable materials, to control the cell physical microenvironment without the necessity of using a separate incubator. The system is compatible with standard imaging laboratory equipment, making it suitable for live or time-lapse imaging. Various adherent cell types have been successfully cultured over a period of at least 5 days, and both stained and imaged *in situ*. The platform also proved to be suitable to control gas tension, *e.g.*, to induce hypoxic conditions inside the microfluidic chip to reproduce *in vivo* conditions. In the future, this chip holder will particularly be applied to screen interactions between cells and biomaterials for regenerative medicine applications inside microfluidic platforms.

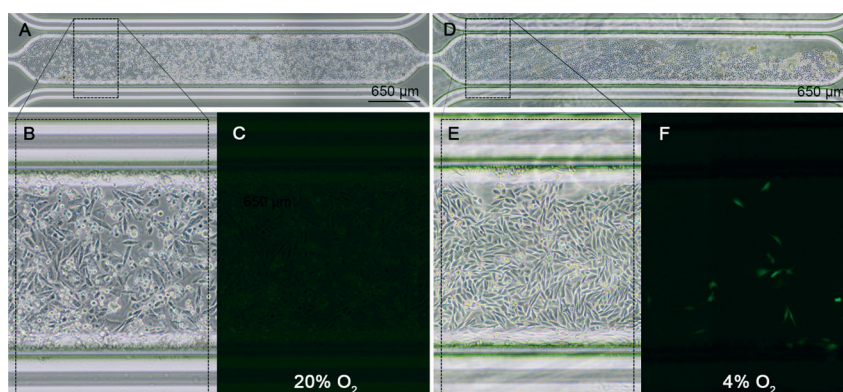


Fig. 4 Cho cells with hypoxia GFP reporter (green) inside a gas-impermeable glass chip, using the chip holder, after 48 h culture under normoxic conditions (20% O_2) on the left (A–C) and after 22 h under hypoxic conditions (approximately 4% O_2) on the right (D–F). For both experiments, the following images are provided: bright-field microscopy stitched overview image (NIKON TS100) of the rectangular chamber, with Cho cells inside (A and D); close-up of one section of the rectangular chamber (B and E); and epi-fluorescence image of the same section of the culture chamber (C and F). After 48 h under normoxic conditions, limited or no fluorescence was observed (C), while under hypoxic conditions increased fluorescence was observed (F), indicating that the gene reporter is activated.

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